1,197,942

PATENT SPECIFICATION

NO DRAWINGS

1,197,942

Inventors: ERNST BRANDL, FRANZ KNAUSEDER and FRANZ REMBOULD

Date of Application (No. 37529/67) and filing Complete Specification: 15 Aug., 1967.

(Patent of Addition to No. 1,111,010 dated 14 April, 1965).

Complete Specification Published: 8 July, 1970.

Index at acceptance:—C2 A(1B, 2D)

International Classification:—C 07 c 35/22; C 12 d 9/04

COMPLETE SPECIFICATION

Process for the production of the Antibiotic Pleuromutilin

We, BIOCHEMIE KUNDL GMBH (formerly Biochemie Gesellschaft mit Beschränkter Haftung), an Austrian Company, of Kundl, Austria, do hereby declare the invention for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for producing the antibiotic pleuromutilin or a pleuromutilin containing mycelium.

A process for producing the antibiotic pleuromutilin from the strain *Clitopilus passeckerianus*. (Pil) Sing. having the NRRL number 3100 is described and claimed in our earlier main patent specification No. 1,111,010. The present invention is concerned with improvements in or modifications of the said process.

According to one aspect of the present invention, there is provided a process for producing pleuromutilin which comprises cultivating a strain of *Clitopilus passeckerianus* (Pil) Sing. having either the NRRL number 3279 or the NRRL number 3100 in an aqueous nutrient medium containing assimilable sources of carbon and nitrogen and extracting pleuromutilin from the mycelium separated from the culture broth.

The present invention also provides a process for producing pleuromutilin or a pleuromutilin containing mycelium, which comprises cultivating a strain of *Clitopilus passeckerianus* (Pil.) Sing. having the NRRL number 3279 in an aqueous nutrient medium containing assimilable sources of carbon and nitrogen.

The above mentioned strain of *Clitopilus passeckerianus* (Pil.) Sing. having the NRRL number 3279, gives a particularly high fermentation yield of pleuromutilin. Cultures of this strain were deposited with the United States Department of Agricultural Research Service under the reference NRRL 3279.

The following Table gives a summary of the productivity of the strain NRRL 3100, described in our main patent specification and the strain NRRL 3279 as compared with the strains described in the literature.

Strain	Yields: mg of isolated pleuromutilin from 1 litre culture
Pleurotus mutilis (Fr.) Sacc.	50[1]
Pleurotus passeckerianus Pilàt	<<50[1]
Drosophila subatrata	small amounts together with drosophilin A C and D (Drosophilin B = pleuromutilin)
Clitopilus passeckerianus (Pil.) Sing. NRRL 3100	2390 crude product corresponding to 1960 100% pure substance
Clitopilus passeckerianus (Pil.) Sing. NRRL 3279	3630 crude product corresponding to 3160 100% pure substance

[*Price 5s. 0d. (25p)*]

According to the literature reference (1) only a small amount of an alcohol-soluble antibiotic is present in the mycelium. It has now been found that in the case of strains 3100 and 3279, the major portion of the antibiotic is present in the mycelium or residue liberated from the culture liquor. This finding constitutes an important technical improvement. The present invention accordingly includes as one of its aspects, extracting the antibiotic from the mycelium separated from the fermentation broth. The process of the present invention thus differs essentially from the methods described in the literature references (1) and (2).

In the following Examples, part (b) of Example 1 and Example 2 illustrate the process of the present invention. Part (a) of Example 1 is given by way of comparison.

1) Kavanagh F., Hervey A. and Robbins W. J., Proc.Nat.Acad.Sci. *37* 570 (1951)
2) Anchel M., J. Biol. Chem. *199* 133 (1952)

EXAMPLE 1

10 litres of fermentation mash from a fermentation charge with *Clitopilus passeckerianus* NRRL 3100 were separated into 8.1 litres of culture filtrate and mycelium by filtration over a pressure suction filter.

a) *Extraction of the antibiotic from the culture filtrate*:

The 8.1 litres of filtrate were extracted twice, with 1.6 litres each of ethyl acetate at natural pH (~6.8), whereby the entire active material passed over to the organic phase. This was dehydrated with Na_2SO_4 sicc., concentrated as far as possible in a vacuum and finally 50 ml of diethyl ether were added thereto. After standing at +3°C for 24 hours the antibiotic was obtained in crystalline form. It was filtered with suction, washed twice with 50 ml of petroleum ether (boiling point 60—80°) and finally dried at room temperature in a vacuum. 5.1 g of crude product, having a degree of purity of 86.2% and corresponding to 4.4 g of pure material, were obtained.

b) *Extraction of the antibiotic from the mycelium*:

The moist mycelium was extracted thrice, each time for 30 minutes, with 3.4 litres of technical acetone (water content ~9%) while stirring vigorously. After combining the extracts and evaporating the acetone in a vacuum, 2.0 litres of aqueous phase were obtained as residue, from which the antibiotic already precipitated. The suspension was then extracted twice with 0.5 litres each of ethyl acetate (DAB 6), the extract was washed once with 1 litre of a 5% Na_2CO_3 solution and subsequently with H_2O until a neutral solution was obtained. Upon subsequent dehydration with Na_2SO_4 sicc. and concentration in a vacuum the antibiotic was obtained in crystalline form. 100 ml. of ether were added to the residue and this was allowed to stand at +3°C for 24 hours. After filtering with suction and washing the crystalline material with 200 ml of petroleum ether (boiling point 60—80°), 18.8 of crude product, having a degree of purity of 81.0% and corresponding to 15.2 g of pure substance, were obtained. A total of 5.1 g+18.8 g=23.9 g of crude product, corresponding to 4.4 g+15.2 g=19.6 g of pure product, was obtained from 10 litres of culture.

EXAMPLE 2

10 litres of fermentation mash from a fermentation charge with *Clitopilus passeckerianus* NRRL 3279 were separated into 8.5 litres of culture filtrate and mycelium by filtration over a pressure suction filter.

a) *Extraction of the antibiotic from the culture filtrate*:

The 8.5 litres of filtrate were extracted twice, with 1.7 litres each of ethyl acetate at natural pH (~6.5). The further working up was affected in a manner analogous to that indicated in the Example for Clitopilus passeckerianus NRRL 3100. 4.8 g of crude product, having a degree of purity of 85.5% and corresponding to 4.1 g of pure substance, were obtained.

b) *Extraction of the antibiotic from the mycelium*:

The moist mycelium was extracted thrice, for 30 minutes each time, with 2.6 litres of technical acetone (water content ~9%) while stirring vigorously. After combining the extracts and evaporating the acetone in a vacuum, 1.5 litres of aqueous phase were obtained, from which the antibiotic already precipitated. The further working up was effected in a manner analogous to that indicated in the preceding Example, whereby 31.5 g of active material, having a degree of purity of 87.3% and corresponding to 27.5 g of pure substance, were obtained. A total of 4.8 g + 31.5 g = 36.3 g of crude product, corresponding to 4.1 g + 27.5 g = 31.6 g of pure product, was obtained from 10 litres of culture.

The obtention of a pleuromutilin containing cake by evaporating the culture mash after fermentation or the mycelium separated from the liquor by filtration, is particularly simple and economical in the case of the strain NRRL 3279.

As discussed in our said main patent specification, it has been found and confirmed by numerous feeding tests that the antibiotic pleuromutilin is a useful additive to animal feeds for improving the utilisation of the feed by the animal.

The above examples illustrate the high content of antibiotic which is present in the mycelium, and the mycelium itself is therefore useful as a pleuromutilin containing mixture for the preparation of a ready-mixed feed composition containing 5% of dry mycelium. The fermentation mash described in the above examples is prepared in a manner analogous to that described in our main specification.

Further tests similar to that described in our main specification illustrating the use of the antibiotic pleuromutilin in feed supplements and ready-mixed feeds are described below. The tests include particulars of results obtained with mycelium containing feeds.

TEST 1

4 groups of 12 pigs in each test group and 8 pigs in the control group.

In the following table the numerals 1 to 4 have the following significances:—

1 = Pleuromutilin 10 ppm

2 = terramycin 20 ppm

3 = mycelium of pleuromutilin fermentations 2.5% of the total ration

4 = control without admixture

	1	2	3	4
Increase during fattening period	743 kg	727 kg	699 kg	494 kg
Food evaluation	1:3.418	1:3.420	1:3.488	1:3.544
Average daily increase per animal	794 g	777 g	747 g	792 g

TEST 2

The test was carried out with 14 pigs as control group (without admixture of antibiotic) and 10 pigs as test group (to which 5% by weight of mycelium was added to the total feed ration).

	Control Group	Test Group
Total increase	878 kg	675 kg
Average increase per animal	62.71 kg	67.5 kg
Average daily increase per animal	836 g	900 g
Feed evaluation	1:3.54	1:3.48
Fattening period	75 days	75 days

The test was repeated with 36 pigs in each group.

	Control Group	Test Group
Total increase	2458 kg	2503 kg
Average increase per animal	68.277 kg	69.528 kg
Average daily increase per animal	699 g	724 g
Feed evaluation	1:3.809	1:3.855
Fattening period	102 days	96 days

TEST 3

In this test 12 young pigs from each of 3 broods of the same age in 2 test groups were secluded together for purposes of habituation 14 days before commencement of the test and were freed from worms in a routinary manner. The male animals were castrated at the age of 4 weeks. Upon commencement of the test at the age of 10 weeks 8 young pigs were selected from each group in such a manner that the sex proportions and initial weight of each of the 2 groups were quite balanced. The feed compositions for the control feed and mycelium containing feed were made up as follows:—

	mycelium	control	mycelium	control
coarse ground barley	57.–	57.–	47.–	47.–
coarse ground corn	—	—	15.–	15.–
coarse ground wheat	7.–	7.–	—	—
coarse ground oats	10.–	10.–	—	—
coarse ground soy beans	10.5	13.–	8.5	11.–
fish meal	3.5	6.–	2.5	5.–
coarse ground rye	—	—	10.–	10.–
low-grade wheat flour	5.–	5.–	10.–	10.–
mixture of mineral matter	2.–	2.–	2.–	2.–
BC 757	5.–	—	5.–	—

In the mycelium containing feed, 2½% of the coarse ground soy beans and 2½% of the fish meal are replaced by mycelium.

The addition of vitamins to the feed was identical in both test groups.

The daily feeding quantities were completely identical by weight for the two groups.

TABULAR SUMMARY

	Mycelium Group	Control Group
Number of animals	8	8
Sex	1 : 1	1 : 1
Average initial weight	27.6 kg	27.9 kg
Average final weight	109.8 kg	99.94 kg
Duration of test	115 days	115 days
Average total increase	82.2 kg	72.04 kg
Average daily increase	715 g	626 g
Total feed consumption	2,170 kg	2,171 kg
Evaluation figure	3.305 kg	3,767 kg

It will be apparent that the mycelium group shows a clearly better evaluation figure and better weight increase than the control group.

TEST 4

Comparative experiments were effected with a ready-mixed feed for poultry containing 10 ppm pleuromutilin.

50 chickens were employed in each group.
In the following table, the numerals 1 to 3 have the following significances:—

1 = Pleuromutilin 10 ppm.

2 = aureomycin 10 ppm

3 = comparison without antibiotic.

	1	2	3
Total increase	60,570 g	58,630 g	55,680 g.
Average increase per animal	1,211 g	1,172 g	1,112 g
Feed evaluation	1 : 2.69	1 : 2.93	1 : 3.18

WHAT WE CLAIM IS:—

1. A process for producing pleuromutilin, or a pleuromutilin containing mycelium, which comprises cultivating a strain of *Clitopilus passeckerianus* (Pil) Sing. having the NRRL number 3279 in an aqueous nutrient medium containing assimilable sources of carbon and nitrogen.

2. A process for producing pleuromutilin, which comprises cultivating a strain of *Clitopilus passeckerianus* (Pil) Sing. having the NRRL number 3279 in an aqueous nutrient medium containing assimilable source of carbon and nitrogen, and extracting pleuromutilin from the mycelium separated from the culture broth.

3. A process for producing pleuromutilin which comprises cultivating a strain of *Clitopilus passeckerianus* (Pil) Sing. having the NRRL number 3100 in an aqueous nutrient

medium containing assimilable sources of carbon and nitrogen, and extracting pleuromutilin from the mycelium separated from the culture broth.

4. A process for producing pleuromutilin or a pleuromutilin containing mycelium substantially as hereinbefore described with reference to Example 2.

5. A process for producing pleuromutilin substantially as hereinbefore described with reference to part (b) of Example 1.

6. Pleuromutilin whenever produced by the process of any preceding claim.

7. An animal feed or feed supplement containing an animal growth rate increasing amount of pleuromutilin produced by the process of any one of claims 1 to 5.

8. An animal feed or feed supplement containing an animal growth rate increasing amount of a pleuromutilin containing mycelium produced by a process according to claim 1 or claim 4.

MATHYS & SQUIRE,
Chartered Patent Agents,
10 Fleet Street,
London, E.C.4.
Agents for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1970.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.